Research Article

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Optimization of degradation of winery-derived biomass waste by Ascomycetes

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Abstract

BACKGROUND: Recently, winery wastes have been classified as pollutants by the European Union and post-product processing is required to lower their hazards. Individual fungal enzymes have limited capacity so mixed fungal degradation combined with pre-treatment can decrease biomass recalcitrance for more efficient breakdown, overcoming these limitations.

RESULTS: Winery biomass degradation by a mixture of *Trichoderma harzianum*, *Aspergillus niger*, *Penicillium chrysogenum* and *P. citrinum* in submerged fermentation and solid state fermentation (SSF) was evaluated. Higher cellulase and β-glucosidase activities were observed in SSF and submerged fermentation, respectively. Statistical modelling predicted the fungal percentage ratio of 60:14:4:2 for *A. niger*: *P. chrysogenum*: *T. harzianum*: *P citrinum* with a substrate:medium ratio of 0.39:1. Under the optimized conditions, cellulase, xylanase and β-glucosidase activities increased to 78.5, 3544.7 and 250.9 U mL^{-1}, respectively. Cellulases and xylanases activities increased more than two-fold. Lignin degradation increased from 8% in submerged fermentation (*P. chrysogenum*) to 17.9% under optimized conditions. Gas chromatography–mass spectrometry (GC-MS) analysis identified 78 significant metabolites, of which stigmasterol, glycerol, maleic acid, xylitol and citric acid were generated by fungal degradation.

CONCLUSIONS: Enhanced degradation of winery-derived biomass was achieved using mixed fungal cultures. GC-MS analysis indicated the production of commercially important metabolites during the process.
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Supporting information may be found in the online version of this article.

Keywords: fungi; fermentation; process optimization; enzyme activities; lignin degradation; metabolomics

INTRODUCTION

Grapes are one of the major global horticultural crops with an estimated production of 69.1 million tonnes during 2012 of which approximately 80% were wine grapes.[1] Australia is an important grape producing region and 1.75 million tonnes of grapes were crushed for wine production during 2012–2013.[2] Like the rest of the world, Australian wineries produce large amounts of biomass waste, amounting to about 50–60% of the total grape crushed during the process.[3,4] In addition, wineries are a high wastewater generator, producing as much as 8 litres per bottle of wine.[5] The problem is ubiquitous and, in recent years, winery wastes have been classified as pollutants by the European Union.[3] Subsequent treatment, specifically post-product processing, is thus required to make winery wastes less hazardous, both in nature and volume.[3] Grape waste consist of grape berries, plant-derived fibres, grape seeds, skin, marcs, stalk and skin pulp. Unlike other agricultural by-products, grape biomass waste has limited use as an animal feed stock due to its poor nutrient value and low digestibility (high concentration of tannins and polyphenols). The polyphenols also slow down microbial utilization of this biomass. A majority of these winery biomass wastes thus end up as toxic landfill.[3]

The major components of grape biomass waste are cellulose, pectins and lignins (including tannins). Fungi belonging to the division Ascomycota, such as *Trichoderma* spp., *Aspergillus* spp. and *Penicillium* spp., are known for their biomass degrading ability and should prove useful in grape biomass degradation. The fungi have been well studied for their ability to produce high levels of cellulase and hemicellulase degrading enzymes.[6,7] Such enzymes also have the potential to be used for generating important molecules such as alcohols, flavonoids, organic acids and phenolics.[8] Thus, there is great potential for biomass degradation as well as utilization of the degradation products. However, the fungal enzymes, depending on their parent fungi, have numerous limitations. One limitation of enzymes such as cellulases is their low comparative activity, on a weight-by-weight basis, compared with many other enzymes such as amylases.[7] Also, the grape biomass degrading enzymes suffer greatly from product inhibition, especially by cellobiose (direct inhibition) and glucose (indirect inhibition), even at low concentrations.[9]

To overcome these limitations, the use of mixed fungal culture degradation has been suggested and reported.[10,11] It is known

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that, apart from satisfactory cellulase production, *Aspergillus* spp. generate highly efficient xylanases and β-glucosidases in exceptional quantities.[12] *Penicillium* spp. can be used for lignin mineralization to enhance the overall degradation process.[13] Moreover, the relative recalcitrant nature of the biomass (due to its complex structure) can be decreased by pre-treatment designed to increase access of enzymes to celluloses and hemicelluloses and the overall surface area of the substrate for more efficient breakdown.[14]

The experiments described herein explore a statistical-based optimization of mixed fungal cultures to achieve enhanced grape biomass degradation. The optimization experiments utilized cultures of *Trichoderma harzianum*, *Aspergillus niger*, *Penicillium chrysogenum* and *Penicillium citrinum* for degradation purposes. The conventional and popular submerged fermentation and the emerging solid state fermentation (SSF) methods were tested. Statistical analysis was performed to generate an optimized mixed fungal 'cocktail' and to achieve a bioreactor-based degradation process with increased biomass degradation. Finally, metabolomic techniques were used to analyse the degradation products and to identify several metabolites of industrial and medicinal interest.

METHODS AND MATERIALS

Grape waste and fungi

Post-fermentation grape waste of *Vitis vinifera* var. Shiraz was obtained from the Australian Wine Research Institute (AWRI), Glen Osmond, South Australia. Fungal cultures of *T. harzianum* and *P. chrysogenum* were obtained from Agpath, Victoria. Fungal cultures of *A. niger* (ATCC 10577) and *P. citrinum* (ACM 4 F) were obtained from the culture collection of Swinburne University of Technology. All fungi were first cultured in Yeast Mannitol broth at 30 °C for 48 h and 0.1 mL (approximately 1×10^7 spores mL^{-1}) of each culture was used for the biodegradation studies.

Degradation process

The American Association of Textile Chemists and Colourists (AATCC) mineral salts iron medium, consisting of NH_4NO_3 (3.0 g L^{-1}), KH_2PO_4 (2.5 g L^{-1}), K_2HPO_4 (2.0 g L^{-1}) $MgSO_4.7H_2O$ (0.2 g L^{-1}) and $FeSO_4.7H_2O$ (0.1 g L^{-1}), was used for all degradation studies with crushed and dried grape waste as the sole carbon source. The pH of this medium was adjusted to 5.6 with 1 mol L^{-1} H_2SO_4.

The grape biomass waste was oven-dried at 70 °C for 96 h and ground using an HR2094 blender (Philips Electronics Australia, North Ryde, Australia). The dried, ground substrate was further oven dried, again for 96 h at 70 °C. The sample (30 g L^{-1}) used for submerged fermentation was sterilized by autoclaving with AATCC mineral medium at 121 °C for 15 min. The sample used for SSF was sterilized at 105 °C for 24 h and used in 1:1 ratio (w/v) with AATCC mineral medium. The submerged fermentation and SSF cultures were then incubated at 30 °C for 1 week with shaking at 200 rpm. To prevent any weight loss and gas exchange, Parafilm® was used to seal the culture flasks.

Design of experiment and statistical analysis

Experimental design and statistical analysis was performed using a full factorial design method developed using Minitab® 16 (Minitab Pty Ltd, Sydney, Australia). One of the simplest methods for complex experimental design, it can analyse multiple factors with each factor containing multiple levels of experimental conditions. In our experiments, we used two factors of fungi (4 types) and methods (2 types) optimizing cellulases, xylanases and b-glucosidases (3 factors), thus yielding a 24 experimental condition model.[15]

For the current experimental design, maximum enzyme activities were the primary responses sought after. However, the responses for reducing sugar and lignin concentrations were also taken into consideration for the experimental design.

Analytical methods

Determination of proteins

Protein determination was performed using the Biuret assay. A 0.2 mL aliquot of appropriately diluted filtrate from the degraded samples was mixed with 0.8 mL Biuret reagent. The mixture was vortexed and incubated at 25 °C for 30 min. The total protein content was determined by absorbance at 546 nm using a Biochrom WPA Biowave II UV/Vis spectrophotometer (Biochrom Ltd., Cambridge, UK) using a quartz cuvette of 1 cm pathlength. Bovine serum albumin was used as the standard for the test.

Determination of reducing sugars

Quantitative determination of reducing sugars in the filtrate of degraded grape waste was performed by dinitrosalicylic acid (DNSA) assay. Grape waste filtrate (100 µL sample) was mixed with 1 mL of DNSA and incubated in a boiling water bath for 5 min followed by cooling in an ice bath in order to quench the reaction before returning the sample to room temperature. The absorbance was taken at 540 nm to determine the concentration of reducing sugars. A glucose gradient was used to derive the standard reducing sugar.

Lignin determination

Lignins were determined as acid soluble lignin (ASL) and acid insoluble lignin (AIL) by the National Renewable Energy Laboratory (NREL) procedure.[16] A sample of dried grape (0.1 g) was incubated in 1 mL 72% H_2SO_4 at 30 °C for 1 h. This was followed by dilution of the acid hydrolysed sample to 4% H_2SO_4. The mixture was then autoclaved at 121 °C for 1 h followed by cooling to room temperature. The supernatant was collected as the ASL fraction after a brief centrifugation. The pellet was rinsed with distilled water and was completely dried at 105 °C for 4 h. The dried sample was weighed as acid insoluble residue (AIR) and was then kept in a muffle furnace at 575 °C for 1 h followed by cooling to room temperature. The weight of this sample was considered as ash. ASL in each sample was determined by the absorbance of the centrifuged filtrate at 320 nm using the equation given below.

$$\%ASL = \frac{ABS \times volume \times Df}{\epsilon \times W_{S1} \times pathlength} \times 100$$

where
ABS = absorbance at 320 nm, volume = volume of total filtrate (30.35 mL), ϵ = absorptivity of biomass at 320 nm (30 L g^{-1} cm^{-1}), W_{S1} = oven dried weight of sample (mg), pathlength = pathlength of the cell (1 cm), and Df = dilution factor.

AIL was determined by the ratio of difference between dry acid insoluble residue and ash to the original dry weight of grape waste as given in the equation below:

$$\%\ AIL = \frac{W_{S2} - W_{S3}}{W_{S1}} \times 100$$

where W_{S1} = oven dried weight of sample (mg), W_{S2} = weight of AIR (mg), W_{S3} = weight of ash (mg).

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The total lignin content was calculated as the cumulative ASL and AIL.

Enzyme assays

Cellulase activity was measured in terms of filter paper activity (FPA) as per IUPAC protocols.[17] 50 mg of Whatman No. 1 filter paper was used as the substrate and was added to 0.5 mL of appropriately diluted filtrate in 0.05 mol L^{-1} sodium citrate buffer (pH 4.8). The mixture was vortexed and incubated at 50 °C for 1 h. DNSA reagent (3 mL) was added to this reaction mixture before boiling for 5 min. The reaction was quenched on ice prior to adding 20 mL de-ionized water. The sample mixture was then vortexed vigorously and allowed to settle for 20 min at room temperature. Absorbance was taken at 540 nm to determine the cellulose activity in terms of FPA. One international unit (IU) of cellulase is defined as the amount of enzyme required to liberate 1 μmol glucose per min under assay conditions.

β-glucosidase activity was determined by p-nitrophenyl-β-D-glucoside (pNPG) assay according to the method of Kovacs *et al.*[18] with slight modifications. Briefly, 1 mL of sodium acetate buffer (0.1 mol L^{-1}, pH 5) and 0.5 mL of 0.02 mol L^{-1} p-nitrophenyl-β-D-glucosidase (pNPG) were added to appropriately diluted enzyme samples in sodium acetate buffer. The mixture was incubated at 50 °C for 5 min. The reaction was terminated by the addition of 2 mL Na_2CO_3 solution (0.2 mol L^{-1}). β-glucosidase activity was determined by measuring the optical density against water at 400 nm. One IU of β-glucosidase is defined as the amount of enzyme required to liberate 1 μmol p-nitrophenol per minute under assay conditions.

Xylanase activity was measured by Highely's method.[19] 1.8 mL of Birchwood xylan (1%) in 0.05 mol L^{-1} Na-citrate buffer was mixed with 200 μL of appropriately diluted enzyme sample and incubated at 50 °C for exactly 5 min. 3 mL DNSA reagent was added to this mixture before boiling for 5 min. The reaction was terminated on ice. Absorbance was taken at 540 nm to determine enzyme activity. One IU of xylanase is defined as the amount of enzyme required to liberate 1 μmol xylose per minute under assay conditions.

Total carbon and nitrogen content

Total nitrogen and carbon content were measured using a carbon and nitrogen analyser (CN 2000, Leco Corporation, St. Joseph, Michigan, USA). Following calibration and drift correction, 100–130 mg of oven dried control and fermented samples were fed into the analyser. Total nitrogen and carbon content was calculated by automated sampling.

Silyl derivatization and gas chromatography–mass spectrometry (GC-MS)

Samples derived from the optimized bioreactor degradation process were further analysed by gas chromatography–mass spectrometry (GC-MS). A 1 mL aliquot of methanol (LC grade, Schar-Lab, Sentemanat, Spain) was added to 40 mg post-degraded freeze dried sample, then vortexed briefly before centrifugation at 572.5 g/4 °C for 15 min. A 50 μL aliquot of the supernatant was then transferred to a fresh tube and dried in an RVC 2–18 centrifugal evaporator at 40 °C/210 g (MARTIN CHRIST Gefriertrocknungsanlagen GmbH; Osterode, Germany). All samples were stored at −80 °C until further use.[20] In order to derivatize the samples for GC-MS analysis, 40 μL methoxamine HCl (2% in pyridine) was added to each sample and incubated for 45 min at 37 °C. To complete the derivatization, silylation was performed by adding 70 μL BSTFA in 1% TMCS. Samples were then incubated for an additional hour at 70 °C. Samples were diluted with 190 μL pyridine, vortexed and centrifuged at 15682 g for 5 min before transferring to GC-MS vials.

GC-MS was performed as previously reported.[21] Briefly, an Agilent 7890B GC oven coupled with a 5977A MS detector (Agilent Technologies, Mulgrave, Victoria, Australia) was used. The GC-MS system was fitted with a 30 m HP-5MS column, 0.25 mm ID and 0.25 μm film thickness. All injections were performed in split mode (1:10) with 1.0 μL volume; the oven was held at an initial temperature of 70 °C for 2 min before increasing to 300 °C at 7.5 °C min^{-1}; the final temperature was held for 5 min. The transfer line was held at 280 °C and the detector voltages at 1054 V. Mass spectra were acquired from 45 to 550 m/z, at an acquisition frequency of 4 spectra s^{-1}. The MS detector was turned off until the excess derivatization reagent was eluted from the column. This ensured that the source filament was not saturated and damaged. Data acquisition and spectral analysis was performed using Agilent MassHunter quantitative analysis program. Qualitative identification of the compounds was performed according to the metabolomics standard initiative (MSI) Chemical Analysis Workgroup using standard GC-MS reference metabolite libraries of Wiley, NIST 11 and NIST EPA/NIH. Chemometric and statistical analysis were undertaken using SIMCA 13, a chemometric software package (Umetrics AG, Umeå, Sweden), and MetaboAnalyst 2.0, an online statistical package (TMIC, Edmonton, Canada). Chromatography peaks were considered significant where Fold Change (FC) was > 2.0, and P-values were ≤ 0.05.

RESULTS AND DISCUSSION

Compositional analysis of grape waste

It is well known that the composition of the substrate strongly influences its degradation efficiency. Various components such as free proteins, sugars and structural components such as cellulose and lignin affect the overall efficiency of degradation, usually providing a barrier to such degradation.[22] In addition, the carbon–nitrogen (C/N) ratio plays an important role in biodegradation. It has been found that the higher the nitrogen content in the substrate, the greater the degradation ability of fungi.[10] In addition, the higher the nitrogen content, especially from organic sources (proteins, for example), the greater the overall enzyme activities.[23] The total compositional analysis of untreated grape wastes used in this study is given in Table 1.

In this study, it was observed that the process of hydrothermal treatment increased the protein content in the medium compared with that in the SSF process (Fig. 1(D)). For example, the observed protein concentration in *T. harzianum* cultured under the submerged condition was 31.9 kg m^{-3}, however, this value decreased under SSF conditions, where it was 11.5 kg m^{-3}. The decrease in total protein content was also reflected sharply in *A. niger* cultures where the protein content decreased from 21.9 kg m^{-3} to 4.5 kg m^{-3}, which was reflected in the decreased β-glucosidase activity. The activity of β-glucosidase also dropped in other cultures as well, as can be observed from Fig. 1(C).

In addition to proteins, lignins also form one of the important determining factors for biomass degradation. Lignins are aromatic polymers and are very complex in their chemical structure (compared with cellulose and hemicelluloses). They form a considerable challenge for degradation, both chemically and biologically.[24] They are also bound to both cellulose and hemicellulose polymers by various cross-linkages,[25,26] thus

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Table 1. General composition of Shiraz grape waste

Component	Content (g per100 g mass) (n = 3)
Proteins	2.22 ± 0.01
Total sugars	1.92 ± 0.18
Lignins	35.96 ± 0.13
Cellulose + Hemicellulose	57.84 ± 0.13
Ash	2.81 ± 0.01

inhibiting the degradation of those polymers. Lignin degradation, or at least its removal and separation from cellulose and hemicellulose, is therefore one of the chief aspects of biomass degradation. Lignin can act as a non-productive inhibitor of cellulolytic enzymes, especially cellulases which tend to have strong binding to lignins.[22] The importance of this property further increases in grape waste due to the high content of lignin-derived products such as phenolics and tannins.[27,28] In this study, it was observed that, compared with the control, *P. chrysogenum* was the only fungus able to degrade lignin to an appreciable extent. Degradation of 4.7% and 2.2% lignin content were seen under submerged and SSF conditions, respectively. None of the other fungi used were found to be lignolytic organisms. However, *Penicillium* spp. has previously been reported to possess minor lignin mineralization abilities, including *P. chrysogenum*, and are known to produce lignin degradation (about 8%) under appropriate conditions.[13] It is quite possible that the lignin degrading ability of *P. chrysogenum* results in an overall increased biomass degradation efficiency, thus requiring considerably less proteins as compared with other fungi under similar conditions.

Autoclaving significantly altered the composition of winery grape waste. Overall, dry mass decreased by about 18% in autoclaved grape waste. It has been reported by numerous authors that biomass undergoes hydrolysis during hydrothermal processing. Although most of the reports have utilized temperatures around 180 °C, the hydrolysis of biomass has been documented to occur even at autoclaving temperatures.[14,29] This occurs due to the increase in ion products of water with a simultaneous decrease in the dielectric constant as the temperature rises. These factors have been reported to significantly increase the solvent capacity of water.[30] This, in turn, increases biomass degrading ability of the system, as observed during course of the mentioned experiment.

Enzyme activities

Cellulase activity was measured as the combined activities of endoglucanases and exoglucanases in FPA. The production of cellulases, β-glucosidases and xylanase is important from the biodegradation perspective. In particular, higher activities of β-glucosidases play a crucial role as they prevent the product inhibition of cellulases.[7,22] To assess the overall activity of these enzymes in all fungal cultures and to generate a better grape biomass degradation method, process optimization was carried out. Generally, about 1:1 cellulase:β-glucosidase activities have been reported to yield higher outputs under SSF conditions[10,31] but this varies across the species and type of substrates.

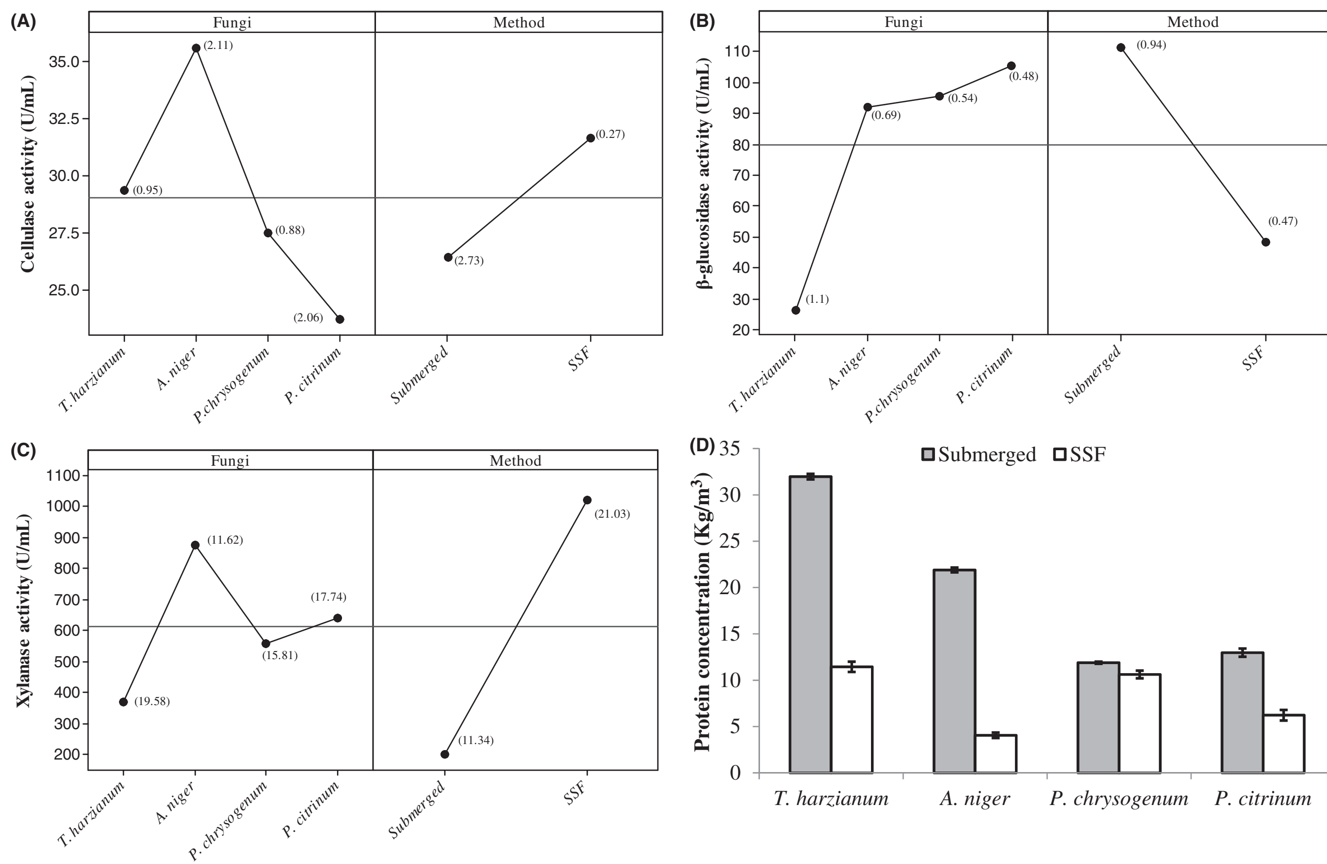

Figure 1. Main effects plot showing the relationship between various fungal cultures across different growth media: (A) in terms of cellulase activity; (B) in terms of xylanase activity; (C) in terms of β-glucosidase activity. The values represented in parentheses denote standard errors. Figure 1(D) represents the protein concentration under submerged and SSF conditions.

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Table 2. ANOVA for cellulases, xylanases and β-glucosidase using adjusted SS for tests

Cellulase	Source	DF	Seq SS	Adj MS	F	P
	Fungi	3	440.6	146.87	42.05	0.001
	Method	1	164.75	164.75	47.17	0.003
	Fungi*Method	2	822.93	274.31	78.53	0
	Error	16	55.89	3.49		
	Total	23	1484.17			
	$S = 1.86896$ $R^2 = 96.23\%$					
Xylanase	Fungi	3	784082	261361	984.86	0.001
	Method	1	4061345	4061345	15304.04	0
	Fungi*Method	2	147477	49159	185.24	0.002
	Error	16	4246	265		
	Total	23	4997150			
	$S = 1.54204$ $R^2 = 99.88\%$					
β-glucosidase	Fungi	3	784082	261361	984.86	0.001
	Method	1	4061345	4061345	15304.04	0
	Fungi*Method	2	147477	49159	185.24	0.002
	Error	16	4246	265		
	Total	23	4997150			
	$S = 16.2904$ $R^2 = 99.92\%$					

The fitted ANOVA model was generated using the data provided in Fig. 1(A), 1(B) and 1(C), and is given in Table 2. High predictive responses (cumulative $Q^2 = 0.929$) were observed across the species and experimental conditions, suggesting good predictive capability of the model used for the enzyme activities of different fungi under varied conditions of growth (Equations (1), (3), (4) and (5), Supplementary material).

The highest activity of cellulase under the full factorial design was noted at 43.7 U mL^{-1}, while those of xylanase and β-glucosidase were observed at 1414 U mL^{-1} and 181.4 U mL^{-1}, respectively. The cellulase activity with the autoclaved substrate varied as per organism, but was found to be generally lower than for SSF. For example, the cellulase activity of *T. harzianum* for the autoclaved substrate was 19.7 U mL^{-1}, lower than SSF (39 U mL^{-1}). A similar trend was seen in the *P. chrysogenum* culture where the activity was 24.1 U mL^{-1}, lower than for SSF (30.7 U mL^{-1}) and for the *P. citrinum* culture where the activity was 15.2 U mL^{-1}, lower than for SSF conditions (27.7 U mL^{-1}). The exception was the *A. niger* culture, where cellulase activity in the autoclaved grape (45.1 U mL^{-1}) was slightly higher than for SSF conditions (28.9 U mL^{-1}) (Table 3). This could be partly due to a higher β-glucosidase activity compared with the grapes applied to SSF. *A. niger* is generally known to possess high β-glucosidase activity.[32] The process of autoclaving is likely to have increased the amount of oligosaccharides and cellobiose in the filtrate, which in turn increased the production of β-glucosidase. This probably led to hydrolysis of cellobiose, one of the product inhibitors of cellulases, during the initial incubation period.

The β-glucosidase activity of *T. harzianum* was observed at 25.8 U mL^{-1} under submerged conditions, comparable with 27.9 U mL^{-1} from SSF. A similar trend was seen with *P. chrysogenum*, where the activity was observed at about 106.3 U mL^{-1} under SSF conditions, as opposite to 84.9 U mL^{-1} under submerged conditions. β-glucosidase activity in the *P. citrinum* culture with autoclaved grapes was 180.1 U mL^{-1}, which was significantly higher than the week 1 SSF culture (30.4 U mL^{-1}). Thus, one of the contrasting outcomes seen in this culture is the comparatively low cellulase activity even with considerable β-glucosidase activity, a result which was unexpected. One plausible explanation for this might be an inherently lower cellulase activity of *P. citrinum*. Conversely, the β-glucosidase activity in the *A. niger* culture with autoclaved grapes was much higher than the SSF substrates. The activity in the autoclaved substrate was found to be 154.9 U mL^{-1} compared with 29.1 U mL^{-1} in SSF. The high β-glucosidase activity was probably related to the comparatively high cellulase activity of *A. niger* on autoclaved substrate.

It is known that β-glucosidases are similar to xylan degrading enzymes, especially xylosidases, with some of them belonging to the same family of GH5 glycoside hydrolases. Their activities are generally dependent on each other and are directly proportional to each other.[33] Confounding this is that cellobiose, the chief substrate for β-glucosidases, is inhibitory towards xylanase activity.[10] In our case, *T. harzianum*, *A. niger*, *P. chrysogenum* and *P. citrinum* displayed xylanase activities for autoclaved grape waste at 38.7 U mL^{-1}, 335.3 U mL^{-1}, 191.1 U mL^{-1} and 234.2 U mL^{-1}, respectively. The activity was much lower than for the SSF substrate, which showed substantially higher xylanase activities of 772.7 U mL^{-1}, 1430.6 U mL^{-1}, 936.8 U mL^{-1} and 1100.8 U mL^{-1}, respectively. This trend of xylanase activity was found to be similar to that of β-glucosidase activity (Table 3). However, the correlation is probably not a causal relationship. The higher activity of β-glucosidase in the SSF cultures, particularly in the *A. niger* culture, presumably prevents accumulation of cellobiose, in turn resulting in higher xylanase activity. The only exception to the relationship between β-glucosidase and xylanase was noticed in *P. citrinum*, which displayed much lower comparative xylanase activity with respect to its β-glucosidase activity for the SSF culture. It has been reported that xylans in biomass act as one of the competitive inhibitors of cellulose enzymes.[22] Thus, a significantly lower activity is likely to occur in organisms with lower xylanase activity, which was mostly consistent with the current experimental outcomes.

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Table 3. Total cellulase, xylanase and β-glucosidase activities (U mL^{-1}) of different fungi under experimental conditions of 'design of experiment'

Method	Fungi	Cellulases (U mL^{-1})	Xylanases (U mL^{-1})	β-glucosidase (U mL^{-1})
SSF	*P. chrysogenum*	31.2	926.9	105.7
SSF	*P. citrinum*	28.1	1046.2	30.1
Submerged	*T. harzianum*	16.7	32.1	26.4
Submerged	*P. citrinum*	21.2	221.0	178.6
SSF	*A. niger*	29.4	1414.0	28.8
Submerged	*A. niger*	39.2	315.1	156.6
SSF	*T. harzianum*	39.8	703.2	29.6
Submerged	*P. chrysogenum*	25.7	156.3	86.0
SSF	*A. niger*	28.5	1414.0	29.2
Submerged	*T. harzianum*	21.2	42.0	23.2
Submerged	*A. niger*	43.7	360.2	153.4
SSF	*P. chrysogenum*	30.8	926.9	106.2
SSF	*P. citrinum*	27.6	1046.2	30.5
Submerged	*P. chrysogenum*	21.2	230.9	83.7
Submerged	*P. citrinum*	16.7	235.9	180.5
SSF	*T. harzianum*	38.4	703.2	24.3
SSF	*P. citrinum*	27.6	1046.2	30.5
Submerged	*T. harzianum*	21.2	42.0	27.8
SSF	*T. harzianum*	38.9	703.2	26.9
SSF	*A. niger*	29.0	1414.0	29.2
Submerged	*P. chrysogenum*	25.7	186.2	85.1
Submerged	*A. niger*	43.7	330.3	154.8
SSF	*P. chrysogenum*	30.3	926.9	107.1
Submerged	*P. citrinum*	21.2	245.8	181.4

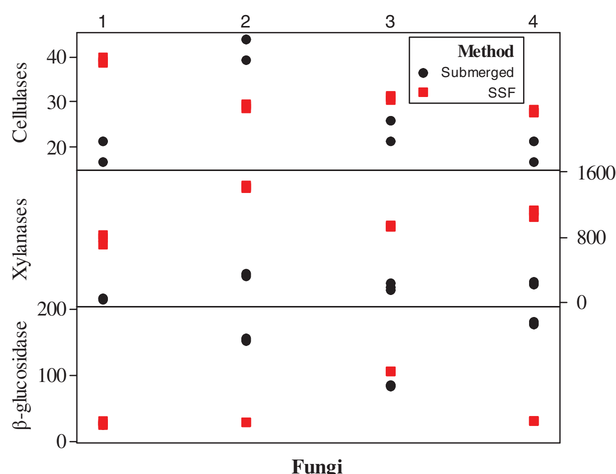

Figure 2. Matrix plot shows the fungal enzyme activities under different conditions. The top X-axis labels of 1, 2, 3 and 4 refer to *T. harzianum*, *A. niger*, *P. chrysogenum* and *P. citrinum*, respectively.

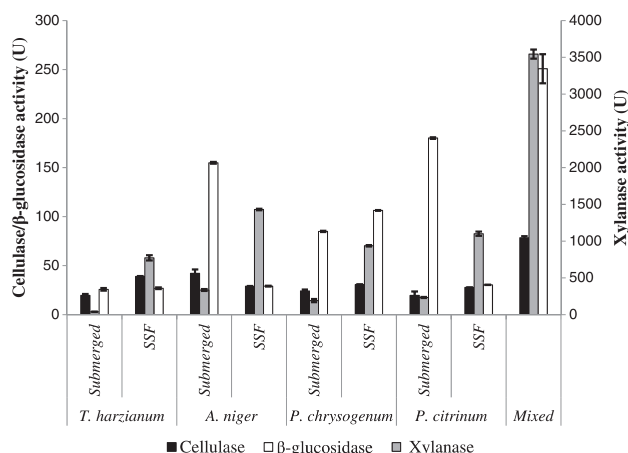

Figure 3. Enzyme activities observed in individual cultures under different conditions compared with that of the optimized mixed culture (n = 3).

The main effects plots and matrix plot generated were able to provide optimum relationships necessary to increase the degradation abilities (Figs 1 and 2). The full factorial design was able to provide the optimum conditions and mix of various fungi for maximizing the degradation of grape biomass. As per prediction of the model, substrate:media ratio was adjusted to 0.3:1 for optimal results. Also, the fungal cultures were selected so as to yield maximum possible output without any considerable enzyme inhibition. The fungal ratio for *A. niger*: *P. chrysogenum*: *T. harzianum*: *P. citrinum* was selected as a percentage ratio of 60:14:4:2.

Grape biomass degradation in bioreactor

The pre-dried and ground grape waste was weighed, adjusted to a 0.3:1 ratio with AATCC medium and sterilized by autoclaving at 115 °C for 10 min in a 3.6 L bioreactor with pH, oxygen, temperature, feed and rpm controls (Infors AG, Bottmingen, Switzerland). The fungal cultures were added at the design levels determined from the Minitab modelling (Tables 2 and 3). The treated grape waste was incubated with this fungal culture for 1 week at 30 °C with constant agitation at 200 rpm. This incubation procedure was identical to that of the individual species, and the results are compared with the results for individual species in Fig. 3.

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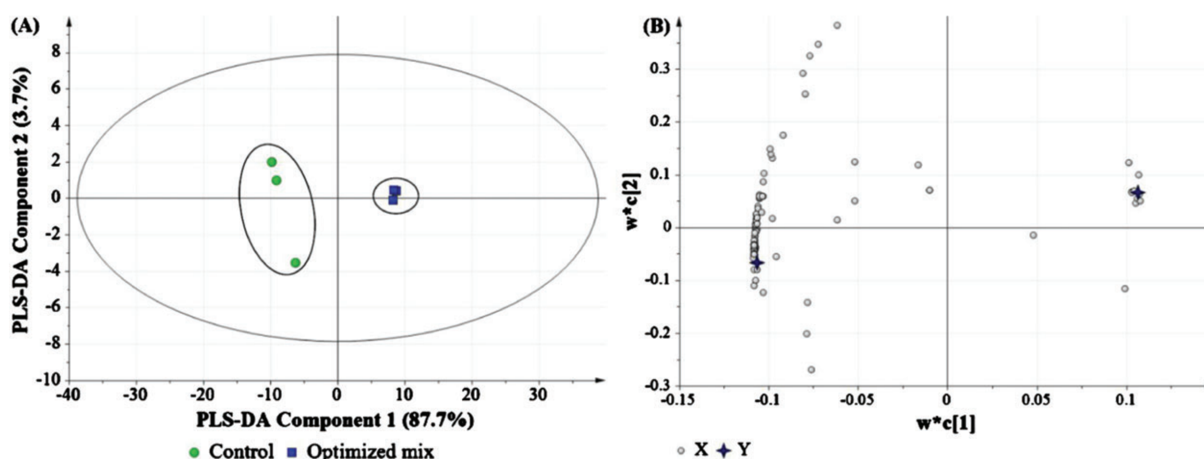

Figure 4. PLS-DA plot (A) and loading plot (B) of degraded Shiraz substrate according to fungal species analysed using GC-MS. Note: the PLS-DA plot eclipse (solid line) represents the 95% confidence interval. The black star labels on the loading plot refer to each fungal group; the circle points refer to each metabolite feature

Cellulolytic enzyme production in bioreactor culture

The activities of all the enzymes were observed to increase under the statistically optimized conditions (Fig. 3). After 5 days of culturing, cellulase activity in the bioreactor was observed at 78.4 U mL^{-1}, more than twice that of *A. niger*, the fungus which displayed the highest cellulase activity by itself (under submerged fermentation conditions). A less dramatic, but still considerable increase was also seen in the activity of β-glucosidase. Under the bioreactor conditions, the β-glucosidase activity was observed at 250.9 U mL^{-1}, considerably higher than that of *P. citrinum*, the fungus which displayed the highest β-glucosidase. The most significant increase was seen in xylanase activity, which was observed as high as 3544.7 U mL^{-1}, also more than twice that of the activity seen during the process optimization stage for individual species (see *A. niger* under SSF conditions). Cellulase activities observed during the bioreactor-mediated mixed fermentation were similar to those of the SSF process at day 15, while the activities of β-glucosidase and xylanase were considerable higher (data not shown).

A significant spike in xylanase activity is probably one of the main reasons for the increase in activities of other enzymes. It is known that the hemicellulases form a complex cross-linking with cellulose and lignins in a biomass.[8] Xylanases, especially those derived from *P. citrinum*, have been reported to be active across a wide pH range and temperature conditions spanning 30–50 °C.[34] Also, due to the close association between cellulases and β-glucosidase, the activity of xylanase is dependent on the activities of these enzymes and vice-versa.

Considerable decrease in lignin content was observed in the degraded substrate, where 17.9% lignin was found to be degraded or mineralized during the process. This rate was considerably higher than 4.7% and 2.2% for the control in the submerged and SSF treatments, respectively.

Metabolic output of mixed fungal degradation

The mixed fungal degradation product was analysed by GC-MS to yield a metabolic profile of *c*. 220 peak features, which is generally expected for a microbial metabolism processes. In order to interrogate the data further, the samples were processed using Partial Least Square-Discriminant Analysis (PLS-DA). PLS-DA is used to analyse large datasets and has the ability to assess linear/polynomial correlation between variable matrices by lowering the dimensions of the predictive model, enabling easy dissemination between the samples and the metabolite features that cause the dissemination.[35]

Of the 220 metabolites, 78 were identified as statistically significant by PLS-DA. Moreover, the change in metabolite concentration during degradation was analysed by one-way ANOVA using Fisher's least significant difference method (Fisher's LSD) and Turkey's honestly significant difference (Turkey's HSD). Numerous metabolites, such as organic acids, alcohols, sugars, sugar acids and amino acids, were observed to be either consumed or generated during the biomass degradation process. Table 4 represents the most significant metabolites analysed by one-way ANOVA.

A volcano plot was further applied to assess the metabolite input and output (Fig. 5). It was observed that a majority of metabolites present in the substrate were consumed by the fungi during the degradation process, while other metabolites were generated. The significantly accumulated metabolites were stigmasterol, maleic acid, xylitol and glycerol. Other metabolites of interest, although statistically less significant, were also generated. These included γ-lactone-d-xylonic acid, d-glucopyranoside, citric acid, ethylene and arabinitol.

CONCLUSIONS

A series of experiments were performed to obtain an optimized protocol for degrading winery biomass waste. Both submerged fermentation and SSF processes were assessed using the fungi *T. harzianum*, *A. niger*, *P. chrysogenum* and *P. citrinum*. Following degradation, the parameters of cellulase, xylanase and β-glucosidase activities, carbon/nitrogen content and lignin content were determined. A full factorial design to predict the optimal ratio of fungi and substrate to medium ratios was employed to demonstrate the effectiveness of a mixed fungi culture.

Enzyme activities and lignin degradation were observed to be considerably enhanced under these optimized conditions, illustrating the significance of a multi-factorial design to mixed culture degradation. In addition to noticeable increases in cellulase and β-glucosidase activities, a greater than two-fold increase was seen in xylanase activity during the mixed fungal fermentation within 5 days as against 7 days in submerged fermentation and SSF. In addition, several metabolites of industrial and medicinal interest

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Table 4. Most significant features generated during the optimized biomass degradation as identified by the volcano plot with their fold change (FC) and *P* values

Peaks	Fold change	*P*-value
Galactonic acid-1,4-lactone	12.031	2.06 e^{-7}
Hexacosanoic acid	711.99	9.89 e^{-7}
FructoseBP	177.47	3.34 e^{-6}
DL-Fucose	3.373	4.07 e^{-6}
Ethyl phosphoric acid	16174	6.89 e^{-6}
Arabinofuranose	27.731	6.95 e^{-6}
alpha-DL-Lyxofuranoside	54.94	6.98 e^{-6}
2-Monopalmitin	9.5698	8.89 e^{-6}
myo- Inositol	49.643	1.06 e^{-5}
Phosphoric acid	143890	1.06 e^{-5}
Oleanolic acid	4170.7	1.15 e^{-5}
Gulose	5.3276	1.17 e^{-5}
D-Fructose O-methyloxime results	229.8	1.20 e^{-5}
α-D-Mannopyranoside	269.66	1.20 e^{-5}
D-Fructose	207.12	1.34 e^{-5}
Glyceric acid	4215.4	1.35 e^{-5}
Heptadecanoic acid	310.16	1.37 e^{-5}
Arabitol	4.0971	1.63 e^{-5}
Tetradecanoic acid	1760	1.80 e^{-5}
Saccharic acid	4.1996	2.09 e^{-5}
Gallic acid	38.383	2.14 e^{-5}
D- Mannitol	119.86	2.17 e^{-5}
Hexadecanoic acid	17611	2.30 e^{-5}
Docosanoic acid	322	2.32 e^{-5}
β-Sitosterol	18.036	2.57 e^{-5}
Maleic acid, 2-methyl- (2TMS) results	0.068538	0.000125
Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)	0.18821	0.000701
Phosphoric acid, bis(trimethylsilyl) 2,3-bis[(trimethylsilyl)oxy] propyl ester	0.19696	0.002067
Glycerol-3-phosphate (4TMS) results	0.19714	0.00207
Stigmasterol trimethylsilyl ether	0.20688	4.94 e^{-5}
1,2,3-Propanetricarboxylic acid, 2-[(trimethylsilyl)oxy]-, tris(trimethylsilyl) ester	0.29432	0.009638
Citric acid (4TMS) results	0.29432	0.009638
D-Xylonic acid, 2,3,5-tris-O-(trimethylsilyl)-, γ-lactone	0.30923	0.003607

such as alcohols, acids and monosaccharides were observed to be produced during the mixed fermentation process.

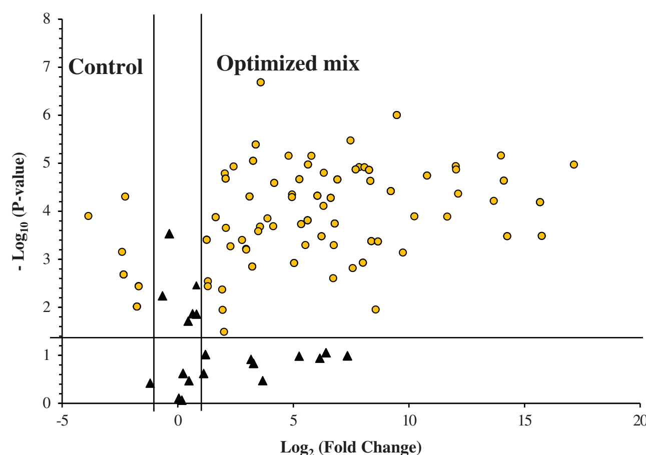

Figure 5. Important features selected by volcano plot with fold change threshold (*x*) 2 and t-tests threshold (*y*) 0.05. Note both fold changes and p-values are log transformed. The further its position away from the (0,0), the more significant the feature is. The yellow circles represent significant metabolites, while the black triangles represent non-significant metabolites.

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Supporting Information

Supporting information may be found in the online version of this article.

REFERENCES

1 OIV. Statistical report on world vitiviniculture 2013. In 36th World Congress of Vine and Wine; 22 January 2014; Bucharest, Romania. Paris, France, ed by Castellucci F. International Organization of Vine and Wine; 2013. 1–28.
2 1329.0-Australian wine and grape industry 2012–2013 [Internet]. Australian Bureau of Statistics. 2013. Available from: http://www.abs.gov.au/AUSSTATS/abs@.nsf/Lookup/1329.0Main+Features12012-13?OpenDocument.
3 Devesa-Rey R, Vecino X, Varela-Alende JL, Barral MT, Cruz JM and Moldes AB, Valorization of winery waste vs. the costs of not recycling. *Waste Manage* **31**:2327–2335 (2011).
4 Gerling C, GRAPES 101 conversion factors: from vineyard to bottle. Appellation Cornell: News from Cornell's Viticulture and Enology Programs [Internet]. (2011) [cited 2014 22 January]; (8). Available from: http://grapesandwine.cals.cornell.edu/cals/grapesandwine/appellation-cornell/issue-8/grapes-101-vineyard-to-bottle.cfm.
5 Christ KL and Burritt RL, Critical environmental concerns in wine production: an integrative review. *J Clean Prod* **53**:232–242 (2013).
6 Brink J and Vries R, Fungal enzyme sets for plant polysaccharide degradation. *Appl Microbiol Biot* **91**:1477–1492 (2011).
7 Klyosov AA (ed), Cellulases of the third generation, in *Biochemistry and Genetics of Cellulose Degradation*, 09 September 1987. Academic Press, Paris, London (1988).
8 Sánchez C, Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnol Adv* **27**:185–194 (2009).
9 Andrić P, Meyer AS, Jensen PA and Dam-Johansen K, Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: I. Significance and mechanism of cellobiose and glucose inhibition on cellulolytic enzymes. *Biotechnol Adv* **28**:308–324 (2010).
10 Brijwani K, Oberoi HS and Vadlani PV, Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. *Process Biochem* **45**:120–128 (2010).
11 Chu Y, Wei Y, Yuan X and Shi X, Bioconversion of wheat stalk to hydrogen by dark fermentation: effect of different mixed microflora

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on hydrogen yield and cellulose solubilisation. *Bioresource Technol* **102**:3805–3809 (2011).
12 Betini JHA, Michelin M, Peixoto-Nogueira SC, Jorge JA, Terenzi HF and Polizeli MLTM, Xylanases from *Aspergillus niger*, *Aspergillus niveus* and *Aspergillus ochraceus* produced under solid-state fermentation and their application in cellulose pulp bleaching. *Bioproc Biosyst Eng* **32**:819–824 (2009).
13 Rodríguez A, Carnicero A, Perestelo F, de la Fuente G, Milstein O and Falcón MA, Effect of *Penicillium chrysogenum* on lignin transformation. *Appl Environ Microbiol* **60**:2971–2976 (1994).
14 Papadimitriou EK, Hydrolysis of organic matter during autoclaving of commingled household waste. *Waste Manage* **30**:572–582 (2010).
15 Montgomery DC, *Design and Analysis of Experiments*. John Wiley & Sons, New York (2008).
16 Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, *et al.*, Determination of structural carbohydrates and lignin biomass. National Renewable Energy Laboratory, 08/07/2011. Report No.: Contract No.: NREL/TP-510-42618 (2011).
17 Ghose T, Measurement of cellulase activities. *Pure Appl Chem* **59**:257–268 (1987).
18 Kovacs K, Macrelli S, Szakacs G and Zacchi G, Enzymatic hydrolysis of steam-pretreated lignocellulosic materials with *Trichoderma atroviride* enzymes produced in-house. *Biotechnol Biofuels* **2**:14 (2009).
19 Highley TL, Carbohydrolase assays, in *Methods in Plant Biochemistry and Molecular Biology*, ed by Dashek WV. CRC Press, Boca Raton, pp. 309–321 (1997).
20 Ng JSY, Ryan U, Trengove RD and Maker GL, Development of an untargeted metabolomics method for the analysis of human faecal samples using *Cryptosporidium*-infected samples. *Mol Biochem Parasit* **185**:145–150 (2012).
21 Beale DJ, Dunn MS, Morrison PD, Porter NA and Marlow DR, Characterisation of bulk water samples from copper pipes undergoing microbially influenced corrosion by diagnostic metabolomic profiling. *Corros Sci* **55**:272–279 (2012).
22 Duarte G, Moreira L, Jaramillo P and Filho E, Biomass-derived inhibitors of holocellulases. *Bioenergy Res* **5**:768–777 (2012).
23 Kim E, Choi H, Kang S, Song K, Han S, Park C, *et al.*, Enhanced production of cellobiose dehydrogenase and β-glucosidase by *Phanerochaete chrysosporium*. *Korean J Chem Eng* **29**:77–81 (2012).
24 Higuchi T, Look back over the studies of lignin biochemistry. *J Wood Sci* **52**:2–8 (2006).
25 Dashtban M, Schraft H and Qin W, Fungal bioconversion of lignocellulosic residues: opportunities and perspectives. *Int J Biol Sci* **5**:578–595 (2009).
26 Fengel D, Ideas on the ultrastructural organization of the cell wall components. *J Polym Sci Pol Sym* **36**:383–392 (1971).
27 Bravo L and Saura-Calixto F, Characterization of dietary fiber and the *in vitro* indigestible fraction of grape pomace. *A J Enol Viticult* **49**:135–141 (1998).
28 González-Centeno MR, Rosselló C, Simal S, Garau MC, López F and Femenia A, Physico-chemical properties of cell wall materials obtained from ten grape varieties and their byproducts: grape pomaces and stems. *LWT - Food Sci Technol* **43**:1580–1586 (2010).
29 Karpe AV, Harding IH and Palombo EA, Comparative degradation of hydrothermal pretreated winery grape wastes by various fungi. *Ind Crop Prod* **59**:228–233 (2014).
30 Goto M, Obuchi R, Hirose T, Sakaki T and Shibata M, Hydrothermal conversion of municipal organic waste into resources. *Bioresource Technol* **93**:279–284 (2004).
31 Chahal DS, Solid-state fermentation with *Trichoderma reesei* for cellulase production. *Appl Environ Microbiol* **49**:205–210 (1985).
32 Shin H-D, Vo T and Chen R, Novel *Aspergillus* hemicellulases enhance performance of commercial cellulases in lignocellulose hydrolysis. *Biotechnol Prog* **27**:581–586 (2011).
33 Pollet A, Delcour JA and Courtin CM, Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families. *Crit Rev Biotechnol* **30**:176–191 (2010).
34 Dutta T, Sengupta R, Sahoo R, Sinha Ray S, Bhattacharjee A and Ghosh S, A novel cellulase free alkaliphilic xylanase from alkali tolerant *Penicillium citrinum*: production, purification and characterization. *Lett Appl Microbiol* **44**:206–211 (2007).
35 Wold S, Sjöström M and Eriksson L, PLS-regression: a basic tool of chemometrics. *Chemomet Intel Lab* **58**:109–130 (2001).

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